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Cloning and Sequencing of *Pseudomonas* Genes Encoding Vanillate Demethylase

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A 2,598-base-pair (bp) *SalI-HincII* DNA fragment has been cloned which codes for vanillate demethylase, the enzyme responsible for the demethylation of vanillate (3-methoxy-4-hydroxybenzoate) to protocatechuate (3,4-dihydroxybenzoate). Complementation and insertional inactivation experiments have shown that this fragment carries two genes (*vanA* and *vanB*) which are predominantly cotranscribed from a promoter upstream of *vanA*. Nucleotide sequencing of the *SalI-HincII* fragment confirmed the genetic data: two open reading frames of 987 and 942 bp were present in the transcribed orientation. These had a very high G+C content in the third base of each codon, which is characteristic of *Pseudomonas* chromosomal genes. Expression of the genes in *Escherichia coli* with the T7 RNA polymerase-promoter system gave rise to two polypeptides of 36 and 33 kilodaltons which could be identified by deletion analysis as the products of *vanA* and *vanB*, respectively. A search of the protein sequence data bank indicated that the *vanB* gene product was related to the ferredoxin family.

Strains of *Pseudomonas* spp. can be quite readily isolated that are capable of degrading aromatic hydrocarbons despite the intrinsic stability of these compounds. With rare exceptions, they do not open the benzene ring unless two hydroxyl groups have been introduced in *cis* into the benzene nucleus (8). Thus, the reactions which prepare the ring for fission tend to be specialized. For example, dealkylation of phenolic ethers is effected by monooxygenases. In these two- or three-component enzyme systems, the electron flow from NADH to the oxygenase is mediated via short electron transport chains (8). The oxygenases themselves fall into two groups: iron sulfur proteins, as in 4-methoxy-benzoate demethylase (2), and cytochrome P450-like enzymes, as in the demethylation of guaiacol (9) and isovanillate (6). It has also been proposed that two proteins may be involved in the demethylation of vanillate (3-methoxy-4-hydroxybenzoate) to protocatechuate (3,4-dihydroxybenzoate) (Fig. 1). Yet purification of the relevant enzyme components has not been successful (5, 24), and their properties and mode of action remain to be characterized.

Vanillate is a key intermediate in the biodegradation of lignin. In chlorinated form, it constitutes one of the major waste products of the paper and pulp industry. To gain further knowledge on the proteins involved in vanillate demethylation, we have cloned the corresponding genes from a *Pseudomonas* sp. This paper describes their isolation and characterization by complementation tests, insertional inactivation, nucleotide sequencing, and expression in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* S17-1 (*pro hsdR hsdM*⁺) contains a modified chromosomally integrated RP4 plasmid which retains its ability to mobilize RSF1010-based vectors, such as pJRD215, to other hosts (26). *Pseudomonas* sp. strain ATCC 19151 was isolated for its ability to utilize sodium dodecyl sulfate (SDS) as a carbon source (15). It is

also able to utilize ferulate and vanillate as sole carbon sources. Plasmid vectors pJRD203 and pJRD215 have been described previously (10, 11). Vector pJRD205 is an improved version of pJRD204 (10) containing the *SalI-XhoI* segment of restriction site bank vector pJRD158 (11) inserted into the *SalI* site of pJRD203. Interposon PHP45 Ω was used to interrupt gene activity and transcription on the cloned DNA (22).

Media and growth conditions. All *E. coli* and *Pseudomonas* strains were routinely grown in L broth or on M9 minimal medium at 37°C. When SDS, vanillate, or other phenolic compounds were used as a carbon source, they were added to the growth medium at a final concentration of 0.2%. Kanamycin and streptomycin were added to the selective medium at final concentrations of 300 μ g/ml and 1 mg/ml, respectively, for the *Pseudomonas* sp.

Enzymes and reagents. All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and *E. coli* Klenow fragment were obtained from Boehringer Mannheim or New England BioLabs and used according to the manufacturer's instructions. The DNA packaging kit and [³²P]dATP (specific activity, >3,000 Ci/mol) were purchased from Amersham Ltd. Phenolic compounds were obtained from Janssen Pharmaceutica, hydrazine was from Kodak, piperidine was from Merck, diethylpyrocyanate was from Sigma, and dimethyl sulfate was from Aldrich Chemical.

Nitrosoguanidine and mutagenesis. The nitrosoguanidine mutagenesis of ATCC 19151 was performed as described by Miller (19). The final concentration of nitrosoguanidine was 200 μ g/ml. The mutants were screened for loss of the ability to use vanillate as a carbon source while retaining the ability to grow on protocatechuate.

Cloning and nucleotide sequencing. All of the recombinant DNA methods used to construct the plasmids or to study the cloned fragments have been described previously (10, 11). The shotgun cloning of the genes involved in vanillate catabolism proceeded in two steps. DNA fragments from an *MboI* partial restriction digest of ATCC 19151 chromosomal DNA were subjected to sucrose gradient (10 to 30%) centrifugation. Fragments of about 30 kilobases (kb) were

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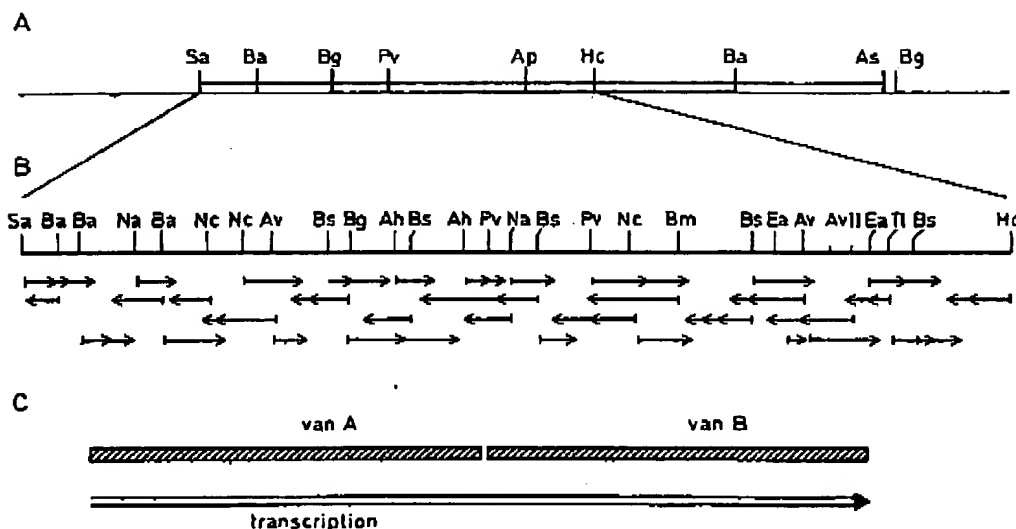


FIG. 2. Restriction map and sequencing strategy for the *Sall*-*HincII* fragment containing the *vanA* and *vanB* genes. (A) Simplified restriction map of the 4.7-kb *Sall*-*AsuII* fragment carrying the *vanAB* genes. The sites shown are those used for subcloning and deletion analysis. The double line indicates cloned *Pseudomonas* DNA, and the single line shows plasmid DNA. (B) Restriction sites used for sequence determination are shown as vertical lines. Horizontal arrows indicate the extent and direction of sequencing. Abbreviations for restriction sites: Sa, *Sall*; Ba, *BamHI*; Na, *NarI*; Nc, *NciI*; Av, *AvaI*; Bs, *BstEII*; Bg, *BglII*; Ah, *AhaI*; Pv, *PvuII*; Bm, *BspMI*; Ea, *EagI*; Av, *AuaI*; Tf, *TfiI*; Hc, *HincII*; Ap, *ApeI*. (C) Positions of the *vanA* and *vanB* genes relative to the physical map of the *Sall*-*HincII* fragment.

agreement with this was the observation that the *BamHI*-*BamHI* (Fig. 2A) subclone showed the same phenotype irrespective of orientation.

The direction of transcription within *Sall*-*AsuII* (Fig. 2A) was determined by in vitro insertional mutagenesis with the 2-kb Ω DNA segment from pHP45 Ω (22) (to perform this experiment, a variant of pJRD205 carrying a deletion of the vector *Sm^r* gene was used). This DNA carries an *Sm^r* gene flanked in both orientations by transcription and translation stop signals. When inserted in a coding region, it terminates RNA and protein synthesis prematurely and thus permits characterization of the functional units. The presence of Ω in the plasmid-borne *Sall* or *BglII* site (Fig. 2A) did not affect complementation, indicating that *van* region expression depends on a promoter situated within the cloned fragment. Insertion of Ω at the *BglII* site in the insert eliminated complementation of both *vanA* and *vanB* mutants. In contrast, insertion at the *ApaI* site eliminated complementation only for *vanB* mutants (Fig. 2A). These results show that the cloned DNA contained the promoter necessary for the expression of the *van* genes. *BglII* is situated within gene A, and *ApaI* is within gene B. Transcription proceeds from *Sall* towards *HincII* and is polycistronic, since both *vanA* and *vanB* mutant complementation was affected by the presence of Ω at the *BglII* site.

Sequencing of the genes for vanillate biodegradation. The nucleotide sequence between the *Sall* and *HincII* restriction sites was determined by using the strategy indicated in Fig. 2B. When possible, both strands were sequenced. Otherwise, at least two different strategies were used on one strand. As with most *Pseudomonas* chromosomal DNA, the sequence of the *van* region was very G+C rich (69.9%). Computer analysis of the nucleotide sequence (Fig. 3) showed two open reading frames in the transcribed orientation (as determined in the previous and following sections).

The first (ORF1) extended from coordinates 259 (ATG) to 1246 (TGA) and had a coding capacity of 329 amino acids. It should be noted that there was a second ATG codon at coordinate 277 (in the same phase). The use of this second ATG, instead of the first, would give a coding capacity of 323 amino acids. The second open reading frame (ORF2), in the transcribed direction, immediately followed ORF1 with an ATG at position 1248 (overlapping the TGA of ORF1) and a TAG stop at position 2190, to give a coding capacity of 314 amino acids. Overlapping or closely adjacent TGA and ATG codons are frequently observed for neighboring cistrons on a polycistronic mRNA (20, 25). Computer analysis (Table 1) showed a very high G+C content in the third base in these open reading frames (ORF1, 90%; ORF2, 92%). This high G+C content is characteristic of bona fide *Pseudomonas* coding sequences (4). Thus, it is most likely that ORF1 and ORF2 are used by the bacterium as genetic information for protein synthesis. This interpretation is supported by the finding that there were sequences resembling ribosome-binding sites upstream of the putative ATG initiator codons (GGGAGGCAAG situated 4 base pairs (bp) from the ATG in position 1248, GACGGCAAG at 11 bp from ATG 259, and GCAACGAA at 4 bp from ATG 277). Both *E. coli* and *P. aeruginosa* 16S rRNAs have similar 3' termini (13), and it is assumed here that *Pseudomonas* sp. strain ATCC 19151 is not greatly different. Table 1 shows the codon usage for the *vanA* and *vanB* genes. They were remarkable for the scarce utilization of codons containing only A and T or having these as the third base.

Open reading frames are common in G+C-rich DNA, and inspection of the nontranscribed strand showed two open reading frames, from base 1655 to 914 and from base 869 to 119, which could encode proteins of reasonable sizes. In contrast with ORF1 and ORF2, these two reading frames did

FIG. 3. Nucleotide sequence of the *SalI*-*HincII* fragment containing the *vanA* and *vanB* genes. The ORF1 and ORF2 open reading frames, corresponding to *vanA* and *vanB*, respectively, have been translated into their predicted amino acid sequences. Only the 5'-3' (sense) DNA strand is shown.

TABLE 1. Distribution of codons in the *vanA* and *vanB* genes

Codon	Amino acid	No. of codons		Codon	Amino acid	No. of codons		Codon	Amino acid	No. of codons		Codon	Amino acid	No. of codons	
		<i>vanA</i>	<i>vanB</i>			<i>vanA</i>	<i>vanB</i>			<i>vanA</i>	<i>vanB</i>			<i>vanA</i>	<i>vanB</i>
TTT	Phe	1	0	TCT	Ser	0	0	TAT	Tyr	0	0	TGT	Cys	0	2
TTC	Phe	10	12	TCC	Ser	7	2	TAC	Tyr	8	5	TGC	Cys	8	10
TTA	Leu	0	0	TCA	Ser	0	0	TAA	End	0	0	TGA	End	1	0
TTG	Leu	0	1	TCG	Ser	0	3	TAG	End	0	1	TGG	Trp	9	2
CTT	Leu	1	2	CCT	Pro	2	0	CAT	His	2	1	CGT	Arg	2	1
CTC	Leu	8	8	CCC	Pro	11	11	CAC	His	7	12	CGC	Arg	17	18
CTA	Leu	0	0	CCA	Pro	0	0	CAA	Gln	2	1	CGA	Arg	1	0
CTG	Leu	18	33	CCG	Pro	7	8	CAG	Gln	12	7	CGG	Arg	6	7
ATT	Ile	1	0	ACT	Thr	0	0	AAT	Asn	1	0	AGT	Ser	0	1
ATC	Ile	22	8	ACC	Thr	9	13	AAC	Asn	6	3	AGC	Ser	6	9
ATA	Ile	0	0	ACA	Thr	0	1	AAA	Lys	0	0	AGA	Arg	0	0
ATG	Met	9	4	ACG	Thr	2	3	AAG	Lys	4	0	AGG	Arg	2	1
GTT	Val	0	0	GCT	Ala	0	0	GAT	Asp	1	0	GGT	Gly	3	2
GTC	Val	5	2	GCC	Ala	31	30	GAC	Asp	20	20	GGC	Gly	21	24
GTA	Val	0	0	GCA	Ala	2	1	GAA	Glu	7	6	GGA	Gly	1	0
GTG	Val	13	16	GCG	Ala	5	10	GAG	Glu	15	11	GGG	Gly	4	3

not have a high G+C content in the third base of the codon and are probably not used to code proteins.

Expression of the vanillate biodegradative genes in *E. coli*. In order to characterize the polypeptide products of the *vanA* and *vanB* genes, expression in *E. coli* maxicells was attempted. No expression was seen, possibly because *Pseudomonas* promoters are generally not recognized by *E. coli* RNA polymerase. To avoid this problem and to specifically label the cloned *Pseudomonas* gene products, use was made of the T7 polymerase-promoter expression system in *E. coli* (27). In this system, T7 polymerase is expressed from the λ p_L promoter under the control of the temperature-sensitive repressor *cl857*. When induced, the T7 polymerase specifically initiates transcription of genes cloned downstream of the T7 $\phi 10$ promoter on a second compatible plasmid. These genes are the only ones to be transcribed when rifampin is added to block *E. coli* RNA polymerase (T7 RNA polymerase being insensitive to rifampin), and their products are easily identified on an SDS-polyacrylamide gel following labeling with [³⁵S]methionine.

Figure 4 shows that when the *SalI-HincII* fragment was cloned and expressed by using the T7 RNA polymerase system, two polypeptides were made. Their sizes were in reasonable agreement with those deduced from the nucleotide sequences. The *vanA* and *vanB* gene products estimated by gel electrophoresis were 37.5 and 33 kilodaltons (kDa), respectively (Fig. 4, lanes 3 and 4), compared with 36.5 and 33.7 kDa estimated from the nucleotide sequence. The use of appropriate deletion derivatives permitted unambiguous identification of the coding regions for the two polypeptides. Thus, the *SalI-BglII* fragment which lacks ORF2 and part of ORF1 did not make the *vanB* polypeptide and synthesized a truncated *vanA* polypeptide (Fig. 4, lane 5). Loss of the ORF1 5' end in the *BglII-AsuII* fragment only permitted *vanB* polypeptide synthesis (Fig. 4, lane 6). No polypeptides were synthesized from the reverse strand (Fig. 4, lane 7). The difference in intensity of the two polypeptides is almost certainly a reflection of their different methionine content; the *vanA* gene product had nine methionine residues, and the *vanB* gene product only had four.

DISCUSSION

In this paper, the cloning and sequencing of two genes involved in the demethylation of a key intermediate in the biodegradation of lignin, 3-methoxy-4-hydroxybenzoate (vanillate), are described. The screening procedure for the

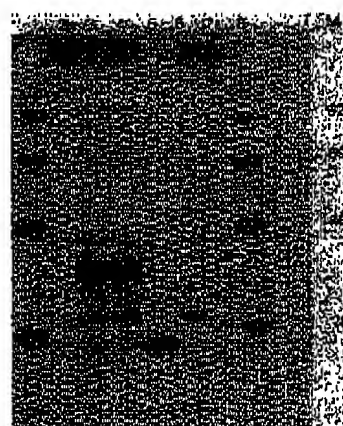


FIG. 4. Polypeptides synthesized in *E. coli* from the *SalI-AsuII* fragment and deleted derivatives. *E. coli* MM294 cultures containing both the pGP1-2 plasmid (T7 RNA polymerase expression plasmid) and the pT7-5 T7 $\phi 10$ promoter plasmid (into which various fragments of the *van* region had been inserted) were induced and labeled with [³⁵S]methionine by the protocol of Tabor and Richardson (27). The [³⁵S]-labeled polypeptides were separated by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS for 3 h at 300 V and visualized by autoradiography. The pT7-5 recombinant plasmids contained various parts of the *van* region. Lane 2, No *van* insert; lane 3, *SalI-AsuII*; lane 4, *SalI-HincII*; lane 5, *SalI-BglII*; lane 6, *BglII-AsuII*. All of these plasmids contained the *van* insert oriented so that it would be transcribed from left to right, as drawn in Fig. 3. Inverting the orientation of the *SalI-AsuII* fragment gave no expression (lane 7). Lanes 1 and 8 contained [¹⁴C]-labeled molecular weight markers (shown in thousands).

recombinant clones was based on the assumption that in *Pseudomonas* sp. strain ATCC 19151, as in other *Pseudomonas* strains (1, 16, 23), vanillate is degraded via protocatechuate and that expression of the cloned genes would enable *van* mutants (defined as capable of growth on protocatechuate but not on vanillate) to grow on vanillate. This assumption was strengthened by the fact that the wild-type strain ATCC 19151 used in the study does not degrade compounds such as guaiacol and catechol, which are intermediates of the vanillate biodegradation pathway of other organisms (21). Moreover, ATCC 19151 does not grow on isovanillate (3-hydroxy-4-methoxybenzoic acid), veratrate (3-4-methoxybenzoic acid), or *o*-, *m*-, or *p*-anisates (2-, 3-, and 4-methoxybenzoic acids), which seems to indicate that the demethylation is a very specific process.

A recombinant clone able to complement six independently isolated *van* mutants was isolated and subcloned on a 4.7-kb *Pseudomonas* DNA fragment. This DNA contains all of the genes necessary for vanillate demethylation, as expression in *Pseudomonas oleovorans* GPO1 (which naturally degrades protocatechuate) conveys to this strain the additional property of using vanillate as a carbon source (data not shown). The intact clone also confers the ability to utilize vanillate on *van* (vanillate utilization) mutants of *Pseudomonas aeruginosa* PAO2175 (from H. Matsumoto). Curiously, deletion clones containing only *vanA* or *vanB* did not complement the mutants. It is probable that interaction between the vanillate monooxygenase and its electron transport protein is highly specific and that one protein cannot be replaced by the equivalent protein from another species. Alternatively, the mechanisms of vanillate degradation may differ between *Pseudomonas* sp. strain ATCC 19151 and *P. aeruginosa*.

The genetic evidence, derived from deletion analysis and insertion inactivation, suggests that two different *van* genes, *vanA* and *vanB*, are located in the left half of the fragment and that these are cotranscribed from left to right. The presence of a secondary promoter for the *vanB* gene was suggested by the delayed complementation of the *vanB* mutants observed when the left-hand end of the cloned DNA was missing. A computer search for sequences similar to the *Pseudomonas* promoter consensus sequence (12) showed that one of them was in fact present 106 bp upstream of the *vanB* start codon (coordinates 1121 to 1142; CTGGC-11 nt-CTGCT). An in-depth study of the regulatory elements carried by the *SalI*-*AsuII* fragment will be published elsewhere.

The characterization of the *vanA* and *vanB* genes, as well as their direction of transcription, was confirmed by DNA sequence analysis, which showed two open reading frames in positions corresponding to those suggested by deletion analysis. The high G+C content of the third base of the codons confirmed the functionality of these open reading frames (4).

The *vanA* and *vanB* genes were expressed in *E. coli* by using a strong promoter from phage T7. In agreement with the nucleotide sequencing results, two polypeptides were visualized, corresponding in size to those predicted by the *vanA* and *vanB* open reading frames. These polypeptides could be correlated with the *vanA* and *vanB* open reading frames by deletion analysis.

The identity of the gene products encoded by the *vanA* and *vanB* genes is not yet known. In *Nocardia* and *Moraxella* spp., the monooxygenases active in the demethylation of isovanillate and guaiacol, respectively, are cytochrome P450s (7, 9, 28). On the contrary, the demethylation of

4-methoxybenzoate by *Pseudomonas putida* is carried out by an iron sulfur protein (3, 8). Comparison of the *vanA* and *vanB* protein sequences with those of all other recorded proteins indicates that *vanB* has similarity to many members of the ferredoxin family. The involvement of a ferredoxin in the vanillate demethylase reaction is quite possible. For example, ferredoxin_{TOX} functions in the electron transport system of the toluene dioxygenase reaction (14). However, it is worth mentioning that the *vanB* gene product (33 kDa) is considerably larger than most ferredoxins, which typically have molecular weights between 8,000 and 15,000. Comparison of the *vanA* gene product with the protein bank failed to give any convincing similarities. Additional biochemical experiments will obviously be needed to obtain a better understanding of the mode of action of the *vanA* and *vanB* proteins.

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